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G_{D3} Vaccines for Melanoma: Superior Immunogenicity of Keyhole Limpet Hemocyanin Conjugate Vaccines[1]

Friedhelm Helling,[2] Ann Shang, Michele Calves, Shengle Zhang, Shunlin Ren, Robert K. Yu, Herbert F. Oettgen, and Philip O. Livingston

Immunology Program and Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, New York 10021 [F. H., A. S., M. C., S. Z., H. F. O., P. O. L.], and Department of Biochemistry and Molecular Biophysics, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298-0614 [S. R., R. K. Y.]

ABSTRACT

Cell surface gangliosides show altered patterns of expression as a consequence of malignant transformation and have therefore been of interest as potential targets for immunotherapy, including vaccine construction. One obstacle has been that some of the gangliosides that are overexpressed in human cancers are poorly immunogenic in humans. A case in point is G_{D3}, a prominent ganglioside of human malignant melanoma. Using an approach that has been effective in the construction of bacterial carbohydrate vaccines, we have succeeded in increasing the immunogenicity of G_{D3} in the mouse by conjugating the ganglioside with immunogenic carriers. Several conjugation methods were used. The optimal procedure involved ozone cleavage of the double bond of G_{D3} in the ceramide backbone, introducing an aldehyde group, and coupling to aminolysyl groups of proteins by reductive amination. Conjugates were constructed with a synthetic multiple antigenic peptide expressing repeats of a malarial T-cell epitope, outer membrane proteins of *Neisseria meningitidis*, cationized bovine serum albumin, keyhole limpet hemocyanin, and polylysine. Mice immunized with these conjugates showed a stronger antibody response to G_{D3} than mice immunized with unconjugated G_{D3}. The strongest response was observed in mice immunized with the keyhole limpet hemocyanin conjugate of the G_{D3} aldehyde derivative and the adjuvant QS-21. These mice showed not only a long-lasting high-titer IgM response but also a consistent high-titer IgG response (predominantly IgG1), indicating recruitment of T-cell help, although the titers of IgM and IgG antibodies following booster immunizations were not as high as they are in the response to classical T-cell-dependent antigens. This method is applicable to other gangliosides, and it may be useful in the construction of immunogenic ganglioside vaccines for the immunotherapy of human cancers expressing gangliosides on their cell surface.

INTRODUCTION

Gangliosides are glycolipid constituents of the cell membrane. The term was coined in 1942 to refer to lipids of the central nervous system that contained sialic acid, to signify their prime location in ganglion cells and their glycosidic nature (1). Their lipophilic component, the ceramide (an amide-linked long-chain sphingoid base and a fatty acid), is thought to be embedded in the outer membrane of the cell membrane lipid bilayer. The carbohydrate portion of the molecule is oriented toward the outside of the cell. Malignant transformation appears to activate enzymes involved in ganglioside glycosylation, resulting in altered patterns of ganglioside expression in tumors such as astrocytoma, neuroblastoma, and malignant melanoma (2). In normal melanocytes, for example, the predominant ganglioside is G_{M3}.[3] Other gangliosides including G_{D3}, G_{M2}, G_{D1a}, and G_{T1b} constitute less than 10% of the total (3). In malignant melanoma, increased expression of G_{D3}, G_{D2}, and G_{M2} has been observed (4, 5), and these gangliosides have therefore been considered potential targets for immunotherapy.

One approach to ganglioside-targeted immunotherapy has been the use of mAbs.[4] Treatment of patients with melanoma or neuroblastoma with mAb recognizing G_{D3}, G_{D2}, or G_{M2} has resulted in tumor regression in some cases (6–9). The other approach has been to immunize patients with ganglioside vaccines in attempts to induce production of ganglioside antibodies by the patients themselves. These attempts have been successful so far only with G_{M2} vaccines. Patients with American Joint Committee on Cancer Stage III malignant melanoma, after complete resection of all tumor, have been shown to produce anti-G_{M2} antibodies in response to vaccination with G_{M2} and *Bacillus Calmette-Guérin* (after pretreatment with low-dose cyclophosphamide to reduce suppressor activity), and the disease-free interval and overall survival were longer in patients producing G_{M2} antibodies (10). G_{D3} and G_{D2}, on the other hand, were found to be only rarely immunogenic when administered in the same way to patients with melanoma (11). Even with the G_{M2} vaccines, the antibody response showed the characteristics of a T-cell-independent response, that is to say, IgM production of short duration, rare conversion to IgG production, and lack of a booster effect (12, 13).

Similar difficulties have been encountered in the development of effective vaccines against bacterial carbohydrate antigens. One approach that has been successful in overcoming these problems is conjugation of the antigen with immunogenic protein carriers. For example, a conjugate vaccine that links the *Haemophilus influenzae* type b capsular polysaccharide to the outer-membrane protein complex of *Neisseria meningitidis* serogroup B was recently shown to induce the production of antibodies and a high rate of protection against invasive disease caused by *Haemophilus influenzae* type b in infants (14), and similar results were reported for a conjugate vaccine using a nontoxic mutant diphtheria toxin as carrier (15).

We have explored this approach in attempts to increase the immunogenicity of melanoma gangliosides. We report here the effects of conjugating G_{D3} with several protein carriers on its immunogenicity in the mouse.

MATERIALS AND METHODS

Gangliosides. G_{M3}, G_{M2} and G_{D1b}, extracted from bovine brain, were provided by Fidia Research Laboratory (Abano Terme, Italy). G_{D2} was made from G_{D1b} by enzymatic cleavage with β-galactosidase from bovine testes (16). G_{D3} (mel) was isolated from human melanoma tissue (17), G_{D3} (bbm) and GT3 were isolated from bovine buttermilk (18), and disialyllactose (G_{D3} oligosaccharide) was isolated from bovine colostrum as previously described (19).

Reagents. HPTLC silica gel plates were obtained from E. Merck (Darmstadt, Germany); 4-chloro-1-naphthol, *p*-nitrophenyl phosphate disodium, and sodium cyanoborohydride were from Sigma Chemical Co. (St. Louis, MO);

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[2] To whom requests for reprints should be addressed, at Immunology Program, Memorial Sloan-Kettering Cancer Center, 425 East 68th Street, Room 820 K, New York, NY 10021.

[3] The designations G_{M3}, G_{M2}, G_{M1}, G_{D3}, G_{D1a} and G_{D1b} are used in accordance with the abreviated ganglioside nomenclature proposed by Svennerholm (40).

[4] The abbreviations used are: mAb, monoclonal antibody; MAP, multiple antigenic peptide; OMP, outer membrane protein; cBSA, cationized bovine serum albumin; ITLC, immune thin-layer chromatography; HPTLC, high-performance thin-layer chromatography; ELISA, enzyme-linked immunosorbent assays; FACS, fluorescence-activated cell sorter; PBS, phosphate-buffered saline; bbm, bovine buttermilk.

methylsulfide was from Aldrich (Milwaukee, WI); cyclophosphamide (Cytoxan) was from Mead Johnson (Syracuse, NY); and QS-21 adjuvant, a homogeneous saponin component purified from *Quillaja saponaria* Molina tree (20), was kindly donated by Cambridge Biotech Corp. (Worcester, MA). It is an amphipathic molecule and was provided as a white powder, forming a clear colorless solution when dissolved in PBS.

Proteins. Poly-L-lysine hydrobromide [MW(vis)3800] was purchased from Sigma, keyhole limpet hemocyanin (KLH) was from Calbiochem (La Jolla, CA), the cBSA-Imject Supercarrier immune modulator was from Pierce (Rockfort, IL), and *Neisseria meningitidis* OMPs were kindly provided by Dr. M. S. Blake (Rockefeller University, New York). MAP YAL-IV 294-I containing four repeats of a malarial T-cell epitope was a gift from Dr. J. P. Tam (Rockefeller University).

Monoclonal Antibodies. Rabbit anti-mouse immunoglobulins conjugated to horseradish peroxidase for ITLC, and rabbit anti-mouse IgM and IgG conjugated to alkaline phosphatase for ELISAs, were obtained from Zymed (San Francisco, CA); anti-G_{D3} mAb R24 was generated in our laboratory (21).

Serological Assays. ELISA were performed as previously described (13). To control for nonspecific "stickiness," immune sera were also tested on plates to which no ganglioside had been added, and the reading was subtracted from the value obtained in the presence of ganglioside. The titer was defined as the highest dilution yielding a corrected absorbance of 0.1 or greater. Immunostaining of gangliosides with mAb or mouse sera was performed after separation on HPTLC silica gel glass plates as previously described (4). Plates were developed in solvent 1 [chloroform:methanol:water (0.25% $CaCl_2$), 50:40:10 (v/v)] or solvent 2 [ethanol:*n*-butylalcohol:pyridine:water:acetic acid, 100:10:10:30:3 (v/v)], and gangliosides were visualized with resorcinol-HCl reagent. Dot-blot immune stains were performed on nitrocellulose strips utilizing purified gangliosides spotted in equal amounts and developed as described before (13).

Immunization. Six-week-old female BALB/c × C57BL/6 F_1 mice (The Jackson Laboratory, Bar Harbor, ME) were given an i.p. injection of cyclophosphamide (15 mg/kg) 3 days before the first immunization and were then assigned to treatment groups. Groups of 4 or 5 mice were given three s.c. injections of a vaccine 2 weeks apart if not otherwise indicated. Each vaccine contained 20 μg G_{D3} or 15 μg disialyllactose and 10 μg QS-21 in a total volume of 0.1 ml PBS. Mice were bled from the retroorbital sinus before vaccination and 2 weeks after the last vaccine injection unless indicated otherwise.

G_{D3} Conjugate Preparation. G_{D3} (2 mg) was dissolved in 2 ml methanol by sonication and cooled to −78°C in an ethanol/dry ice bath. Ozone was generated in an ozone generator (Del Industries, San Luis Obispo, CA) and was passed through the sample for 30 min under vigorous stirring (22, 23). The excess of ozone was then displaced with nitrogen over a period of 10 min. Methylsulfide (100 μl) was added (24), and the sample was kept at −78°C for 30 min and then at room temperature for 90 min under vigorous stirring. The sample was dried under a stream of nitrogen and monitored by HPTLC. The long-chain aldehyde was separated by adding *n*-hexane (2 ml) to the dry sample, followed by sonication for 5 min and centrifugation at 2000 × *g* for 15 min. The *n*-hexane was carefully drawn off and discarded, and the sample was dried under a stream of nitrogen. Cleaved G_{D3} and native G_{D3} were separated by HPLC (Waters, System 501, Milford, MA) utilizing a C_{18} reversed-phase column (10 x 250 mm; Rainin Instruments, Ridgefield, NJ). Gangliosides were eluted with a linear water-acetonitrile gradient and monitored at 214 nm, and the fractions were analyzed by HPTLC. Fractions that contained cleaved G_{D3} were combined and evaporated at 37°C with a Rotavapor (Büchi, Flawils, Switzerland). Cleaved G_{D3} (1.5 mg), 1.5 mg protein carrier in PBS, and 2 mg sodium cyanoborohydride were incubated under gentle agitation at 37°C for 48 h. After 16 h 1 mg sodium cyanoborohydride was added. The progress of coupling was monitored by HPTLC. G_{D3}-protein conjugates did not migrate in solvent 1 and solvent 2 but remained at the origin as a resorcinol-positive band. The mixture was dialyzed across 5000 molecular weight cutoff dialysis tubing with three changes of PBS (4 liters each), at 4°C for 48 h, and passed through an Extractigel detergent-removing gel (Pierce, Rockfort, IL) for final purification of unconjugated G_{D3}. The samples were lyophilized, and their protein and ganglioside content was determined by BioRad protein assay and by neuraminic acid determination according to the method of Svennerholm (25).

Disialyllactose Conjugate Preparation. Disialyllactose was isolated from bovine colostrum as described previously (19). The carbohydrate was attached to protein by reductive amination (26). Disialyllactose (10 mg) was incubated with 2 mg proteins in 2 ml PBS for 14 days at 37°C after sterile filtration. Sodium cyanoborohydride (2 mg) was added at the beginning, and 1 mg was added every 3 days. The coupling was monitored by HPTLC in solvent 2. The disialyllactose conjugates were purified by dialysis across 5000 molecular weight cutoff dialysis membrane with three changes of PBS (4 liters each) at 4°C for 48 h, followed by lyophilization. The protein and neuraminic acid content was determined as described above. Disialyllactose was also conjugated to proteins according to the method described by Roy and Laferrière (27). During this procedure *N*-acroloylated glycopyranosylamine derivatives of the oligosaccharide were formed first, followed by conjugation via Michael addition to amino groups of the protein. Purification and determination of protein and neuraminic acid content were performed as described above.

Determination of Antibody Subclasses. Determination of antibody subclasses was performed by ELISA using subclass-specific rabbit anti-mouse immunoglobulins IgG1, IgG2a, IgG2b, IgG3, and IgA (Zymed, San Francisco, CA). Alkaline phosphatase-labeled goat anti-rabbit IgG served as the signal-generating reagent.

FACS Analysis of Mouse Antisera. A single cell suspension of the melanoma cell line SK-MEL-28 was obtained after treatment with 0.1% EDTA in PBS followed by passage through a 26½-gauge needle. Cells (3×10^5) were incubated with 40 μl of 1:20 diluted post- or preimmunization serum for 30 min on ice. The cells were washed three times with 3% fetal calf serum in PBS. Thirty μl of diluted (1:50) fluorescein isothiocyanate-labeled goat anti-mouse IgG (Southern Biotechnology Associates Inc., Birmingham, AL) were added as secondary antibody, followed by incubation on ice for 30 min. Cells were washed three times as above and resuspended in 500 μl 3% fetal calf serum in PBS and analyzed by flow cytometry (FACScan, Becton Dickinson, San Jose, CA).

RESULTS

Preparation and Characterization of G_{D3}-Protein Conjugates. G_{D3} (bbm) in methanol was selectively cleaved with ozone at the C4-C5 double bond in the ceramide portion. It is assumed that methoxyperoxides are formed as intermediate products (24), and therefore methylsulfide was added as a reducing agent. The result of the cleavage was a G_{D3} derivative with an aldehyde functional group in the position of the former double bond in the ceramide portion (Fig. 1). Cleaved G_{D3} migrated slower than native G_{D3}, and formed double bands because the ceramide contained unsaturated fatty acids that were cleaved simultaneously (see Fig. 1, *inset*). Densitometric analysis of HPTLC plates showed that more than 70% of G_{D3} (bbm) was cleaved by this procedure. Preliminary experiments involving longer ozone treatment had similar results, indicating that 30% of G_{D3} from this source consists of sphinganine or phytosphingosine analogues that contain no ozone-cleavable ceramide double bond. Cleavage at −78°C with ozone treatment up to 1 h (depending on the amount of G_{D3} used) was found to be optimal. Cleaved G_{D3} persisted only in acidic and neutral phosphate buffers for up to 72 h, but with the formation of increasing amounts of oligosaccharide due to β-elimination reactions [which have been shown to occur much faster at alkaline pH (23)]. The decreased hydrophobicity of cleaved G_{D3} compared to native G_{D3} allowed its separation by HPLC on C_{18} reversed-phase columns. Utilizing isocratic elution with a linear water-acetonitrile gradient, cleaved G_{D3} was recovered first, and uncleaved G_{D3} was eluted in later fractions. The incubation of cleaved G_{D3} with proteins resulted in the formation of Schiff bases between the cleaved ganglioside and ε-aminolysyl groups. They were reduced with sodium cyanoborohydride to form stable secondary amine bonds (28). The reaction was monitored by HPTLC, which showed a decreasing ratio of the cleaved G_{D3} to a resorcinol positive band at the origin, indicating the formation of neoglycoconjugates. The reaction was generally completed after incubation for 48 h at 37°C. Disialyllactose was readily remov-

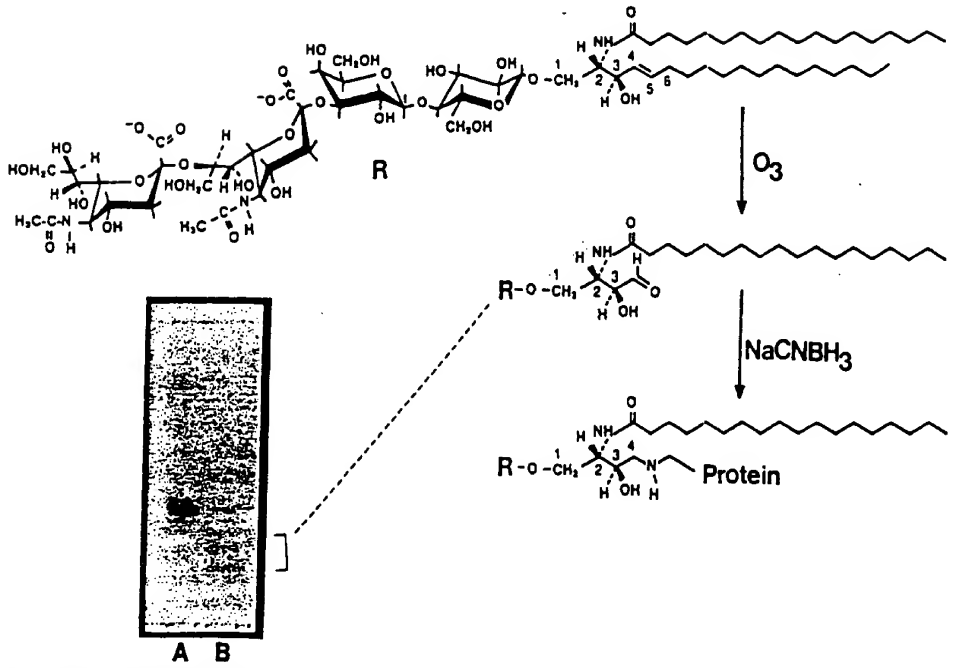

Fig. 1. Synthesis of G_{D3} protein conjugates after ozone cleavage and reductive amination. *Inset*, HPTLC of G_{D3} before (*A*) and after (*B*) ozone cleavage.

ible by dialysis, and the excess of cleaved G_{D3} was removed by passage through a detergent-removing column. The degree of coupling was determined by sialic acid and protein determinations. The weight ratio of G_{D3} to proteins in the different conjugates, shown in Table 1, depended on the accessibility of lysine groups in the proteins. The average yield of G_{D3} coupled to proteins was 30%. G_{D3} conjugates prepared in this way were reactive with anti-G_{D3} mAb R24 by Western blot analysis, although the G_{D3}-aldehyde derivative itself was not reactive by ITLC (data not shown).

Oligosaccharide Conjugation. The carbohydrate part of G_{D3}, disialyllactose, was coupled to proteins utilizing two methods. The first method, reductive amination, resulted in conjugation of the open ring form of the glucose to proteins (26). The method required a long incubation of the oligosaccharide with proteins, and the yield was less than 20%. In the second method (27), involving *N*-acroloylation of the terminal glucose, the oligosaccharide was coupled to proteins with a closed ring formation. None of these oligosaccharide conjugates showed reactivity with mAb R24 by Western blot analysis (data not shown).

Induction of a Serological Response against G_{D3} by Immunization with G_{D3}-Protein Conjugates. All vaccines were well tolerated. Mice were observed for at least 6 months, and neither acute nor systemic toxicity was detected. The serological response to immunization with G_{D3} or G_{D3}-protein conjugates, using QS-21 as adjuvant, is shown in Table 1. QS-21 was used because we had previously demonstrated its superiority over other adjuvants with another carbohydrate antigen-KLH conjugate vaccine (29). In ELISA, preimmunization sera showed no IgM or IgG antibodies reactive with G_{D3}. Immunization with unconjugated G_{D3} did not induce the production of G_{D3} antibodies. Immunization with G_{D3} conjugates, on the other hand, was effective in inducing antibody production. Of the five proteins used in the preparation of the conjugates, KLH showed the

Table 1 *Antibody response to immunization with different vaccines containing G_{D3} or disialyllactose conjugated to carrier proteins*

Vaccine + QS-21	No. of mice	G_{D3}:protein weight ratio[a]	Reciprocal ELISA peak titer against G_{D3}	
			IgG	IgM
G_{D3}	5		0 (5)	20 (3), 0 (2)
G_{D3}/KLH[b]	5	0.33	0 (5)	160, 40, 20 (3)
G_{D3}-KLH[c]	14	0.69	10,240 (2), 5,120 (2), 2,560 (3), 1,280 (2), 80 (2), 40 (2), 0	2,560, 1,280 (2), 640, 320 (3), 160 (2), 80 (3), 20, 0
G_{D3}-cBSA[c]	15	0.77	2,560 (2), 320 (2), 160, 80 (2), 40 (4), 20 (2), 0 (2)	80 (2), 40 (2), 20 (7), 0 (4)
G_{D3}-OMP[c]	15	0.93	2,560, 80 (4), 20 (3), 0 (7)	1,280, 320 (2), 160 (7), 80 (4), 40
G_{D3}-MAP[c]	10	1.0	40, 0 (0)	160 (2), 40 (4), 20 (3), 0
G_{D3}-Polylysine	10	ND	0 (10)	320, 160 (4), 80, 40, 20 (2), 0
Disialyllactose-KLH[d]	4	0.055	0 (4)	160 (3), 80
Disialyllactose-cBSA[d]	4	0.16	20, 0 (3)	40, 20 (3)
Disialyllactose-KLH[e]	4	0.25	20, 0 (3)	40 (2), 0 (2)
Disialyllactose-cBSA[e]	4	0.34	0 (4)	0 (4)
Disialyllactose-Polylysine	5	ND	0 (5)	80 (3), 40 (2)

[a] Protein and ganglioside content were determined by BioRad protein assay and by neuraminic acid determination according to the method of Svennerholm (25).
[b] G_{D3} and KLH were mixed prior to immunization.
[c] G_{D3} was covalently attached to proteins prior to immunization after ozonolysis as described in "Materials and Methods."
[d] Disiallylactose was conjugated to KLH and cBSA by reductive amination according to the method of Gray (26).
[e] Disiallylactose was conjugated to KLH, cBSA, and poly-L-lysine after *N*-acroloylation and Michael addition according to the method of Roy and Laferière (27).

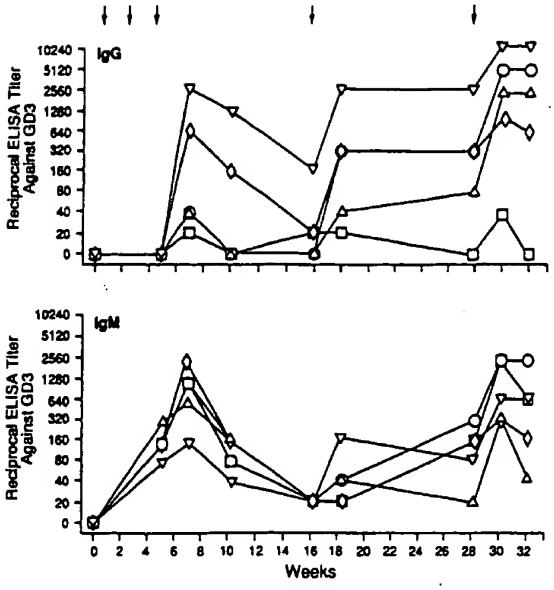

Fig. 2. Time course of G_{D3} antibodies induced in representative mice immunized with G_{D3}-KLH and QS-21 vaccine. Each symbol represents an individual mouse. Arrows, time of vaccination.

strongest immunogenicity, resulting in a median titer of 1:320 for IgM and 1:2560 for IgG antibodies. The specific isotype profile was determined with subclass-specific secondary rabbit anti-mouse antibodies. Antigen-specific antibodies were found to be predominantly of the IgG1 subclass. Antigen-specific IgG2a and IgG2b antibodies were found only in traces, and no IgG3 or IgA antibodies were detected.

In contrast to immunization with G_{D3} conjugates, immunization with G_{D3}-oligosaccharide conjugates induced only a weak IgM response to G_{D3} and no IgG response.

Sequential IgM and IgG antibody titers against G_{D3} for five mice immunized with G_{D3}-KLH and QS-21 are shown in Fig. 2. IgM titers peaked 2 weeks after the third vaccination and declined by the time of the first booster immunization at week 16. The first booster immunization had no significant impact on IgM titers, but the second booster immunization at week 28 increased IgM titers to the peak level seen after the third vaccination of the initial series. IgG titers also rose up to 2 weeks after the third vaccination and decreased by the time of the first booster vaccination but rapidly increased after the booster to previous peak titers. IgG titers remained at this level for 10 weeks, with a further increase after the second booster in most mice. The evidence for a secondary immune response after the booster immunization was therefore equivocal. The response was clearly more rapid than after the initial immunization and lasted longer, but the increase in titer was not comparable to booster responses seen with classical T-cell-dependent antigens.

Specificity of the Serological Response to Immunization with G_{D3}-Protein Conjugates. The specificity of the serological response to immunization with G_{D3}-protein conjugates and QS-21 was analyzed by dot-blot immune staining and ITLC. An example of dot-blot immune stain analysis is shown in Fig. 3. Preimmune sera and immune sera showing high G_{D3}-antibody titers in ELISA were tested on nitrocellulose strips that had been spotted with G_{D3} (bbm) or G_{D3} (mel) and purified structurally related gangliosides: G_{M3}, G_{D2}, G_{D1b}, and G_{T3}. As expected on the basis of the ELISA results, preimmune sera showed no reactivity. In contrast, sera obtained after immunization with KLH conjugates of G_{D3}-ganglioside reacted with G_{D3} (bbm) (the immunogen) or G_{D3} (mel), but not with the other gangliosides except G_{T3} in some cases, a pattern also seen in tests of the mouse monoclonal IgG3 antibody R24, the reagent by which high cell surface expression of G_{D3} on human melanoma cells was first defined (20). The same specificity pattern was seen in dot-blot immune stain tests of sera from mice immunized with other G_{D3}-protein conjugates, the only exception being high-titer sera (by ELISA) from mice immunized with G_{D3}-cBSA, which showed no reactivity with G_{D3} or the other gangliosides.

ITLC permits specificity analysis of ganglioside antibodies in tests on tissue extracts. Examples of tests with high-titer sera from mice immunized with G_{D3}-KLH and QS-21 are shown in Fig. 4. The sera were tested at a dilution of 1:150 on ganglioside extracts of human brain, neuroblastoma, and melanoma, as well as G_{D3} (bbm) that had been used for immunization. The figure shows HPTLC ganglioside patterns of these reagents after staining with resorcinol, as compared with the patterns of reactivity exhibited after exposure to sera from immunized mice or mAb R24. As can be seen in the resorcinol-stained panel, the predominant gangliosides in the brain tissue extract are G_{M1}, G_{D1a}, G_{D1b}, and G_{T1b}, whereas the neuroblastoma extract shows G_{D2} and G_{M2} in addition, and the melanoma extract contains mainly

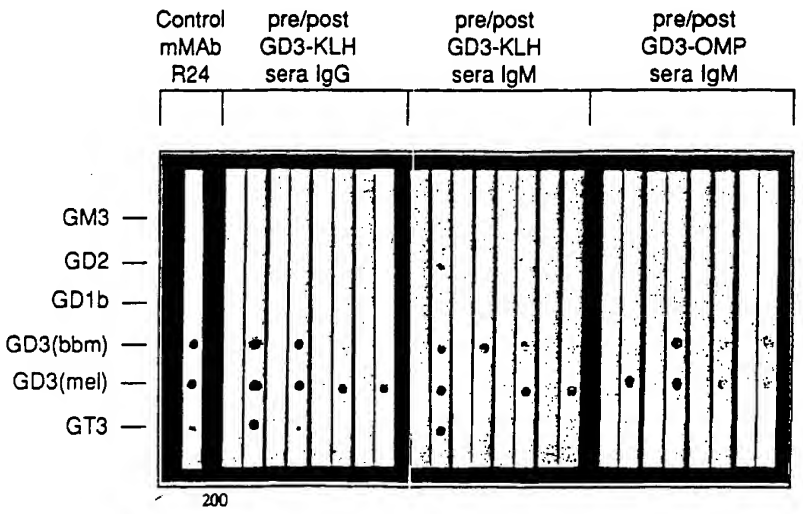

Fig. 3. Dot-blot immune stain assay for IgM and IgG antibodies in sera of mice immunized with G_{D3}-KLH and G_{D3}-OMP conjugates and QS-21. Antigen standards were applied to nitrocellulose strips in equal amounts (0.5 μg) and were allowed to react with pre/postimmunization serum from individual mice.

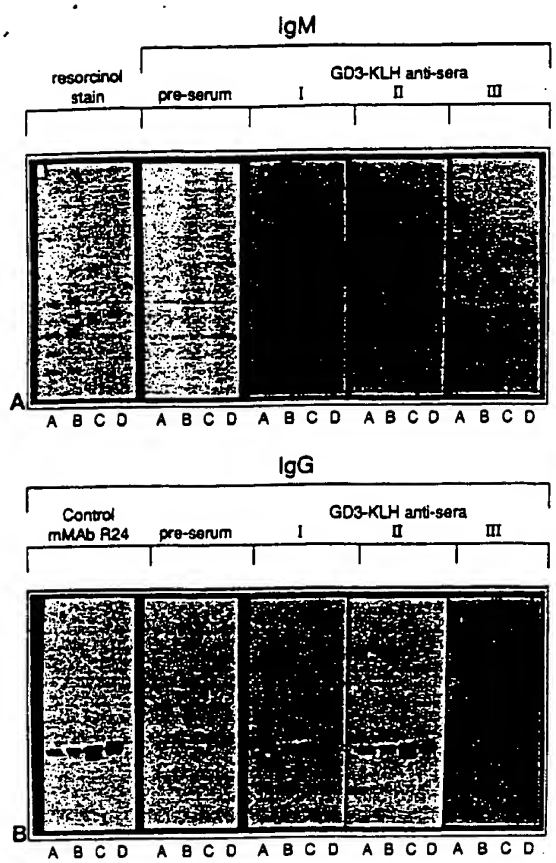

Fig. 4. Immune thin-layer chromatograms of three representative mouse sera after accination with G_{D3}-KLH conjugate and QS-21. IgG and IgM antibodies in pre- and stvaccination sera and anti-G_{D3} mMab R24 were tested on human brain gangliosides), neuroblastoma gangliosides (*B*), melanoma gangliosides (*C*), and G_{D3} (*D*) (bbm). angliosides were chemically stained with resorcinol-HCl reagent to demonstrate the nglioside composition of each sample.

$_{D3}$ and G_{M3}. Reactivity of IgG antibodies in postimmunization sera, s well the reactivity of IgG3 mouse monoclonal antibody R24, was stricted to G_{D3} (Fig. 4*b*). The high-titer IgM antibodies, on the other and, showed weak cross-reactivity with other gangliosides and sul- itide in the brain extract (Fig. 4*a*).

Sera from mice immunized with other G_{D3} conjugates were tested the same way (at lower dilution) and showed the same specificity ith the exception, again, of high-titer sera from mice immunized ith G_{D3}-cBSA, which showed no ganglioside reactivity (data not own).

Cell Surface Reactivity of Immune Sera Determined by FACS nalysis. Sera from mice were tested for binding to cells of the elanoma cell line SK-MEL-28, a cell line known to express cell rface G_{D3}. A representative example of a FACS analysis utilizing a uorescein isothiocyanate-labeled secondary goat anti-mouse anti- ody is shown in Fig. 5. Sera before and after immunization with $_{D3}$-KLH and QS-21 were tested. Preimmunization serum stained 8% f the target cells, postimmunization serum 92%.

ISCUSSION

Conjugation of poorly immunogenic antigens to highly immuno- enic carrier molecules is a well-known approach to augmenting nmunogenicity. Ganglioside molecules are so small, however, that linkage to carrier molecules without affecting the relevant antigenic epitopes is difficult. We have shown previously that modifications of G_{D3} in its carbohydrate portion (*i.e.*, conversion of sialic acid carboxyl groups to amides or gangliosidols or lactones) results in markedly increased immunogenicity. However, antibodies produced in response to these G_{D3} derivatives show no cross-reactivity with native G_{D3} (11, 30). Covalent attachment of proteins to the sialic acid molecules of G_{D3} was therefore not attempted in the present study. Our initial approach involved conjugation of G_{D3} oligosaccharide (disialyllactose) via the terminal glucose in open- or closed-ring configuration to KLH or polylysine, but these conjugates were not recognized by the anti-G_{D3} mAb R24 or by mouse antisera to G_{D3}, and mice immunized with the conjugates did not produce G_{D3} antibodies. Subsequently, we coupled G_{D3} to proteins via its ceramide portion without alteration of the carbohydrate moiety. The ceramide was cleaved with ozone at the double bond of the sphingosin base, and coupling to proteins was accomplished by reductive amination. Cleavage of gangliosides by ozonolysis and subsequent conjugation with proteins by this method has not been described, and it has been generally assumed that the aldehyde intermediates of gangliosides would be unstable. Fragmentation, initiated by hydroxy ions under alkaline conditions, has been reported. Migration of the double bond would result in β-elimination, causing release of the oligosaccharide moiety (22, 31). We found, however, that the aldehyde was sufficiently stable at neutral pH to permit Schiff base formation with amino groups of proteins, so that β-elimination was not a major problem. The overall yield was 30%. These G_{D3} aldehyde-protein conjugates showed reactivity with G_{D3} antibodies by Western blot analysis, indicating that the immunodominant epitopes were intact in these G_{D3} conjugates. However, reactivity of the G_{D3}-aldehyde derivative with mAb R24 by ITLC could not be shown. This may be due to its relatively unstable nature, resulting in β-elimination and release of oligosaccharide during the immune stain incubation period, or simply to the fact that the G_{D3}-aldehyde derivative may not adhere to the thin-layer plate sufficiently for serological detection.

Earlier studies describe oxidative ozonolysis of the glycosphingolipid olefinic bond, resulting in a carboxyl group that could be conjugated with carbodiimide to NH_2 groups of modified glass beads, agarose gel, or other macromolecules (32, 33). This method, however, is of limited use for the conjugation of gangliosides to carrier proteins because it requires acetylated, methyl ester derivatives of gangliosides to avoid coupling via the sialic acid carboxyl group. Deacetylation after conjugation under basic conditions is necessary, conditions most proteins cannot be exposed to without degradation.

Once the conjugation method was established, several protein carriers were considered, based on previous work by others. Lowell *et al.* (34) described an elegant system that resulted in high-titer antibody responses as a consequence of anchoring bacterial carbohydrate and peptide antigens via a synthetic, hydrophobic foot in OMPs of *Neisseria meningitidis* (35). This system was directly applicable to gangliosides because of their amphipathic nature. In previous studies, we adsorbed gangliosides onto OMP by hydrophobic interaction, and we were able to induce high-titer IgM responses (36). Covalent attachment was utilized in the current study, but G_{D3}-OMP conjugates induced only occasional IgG responses, and the IgM response was not increased. Conjugation with cationized BSA, which has been reported to be a potent carrier for protein antigens (37), resulted in high-titer IgG antibodies detected by ELISA, but immune stains indicated that the response was not G_{D3}-specific. Another appealing carrier is the MAP system described by J. P. Tam (38, 39). MAPs consist of four or eight dendritic peptide arms, containing B- and T-cell epitopes, attached to an oligomeric branched lysine core. The antibody response to peptides was dramatically increased when these constructs were

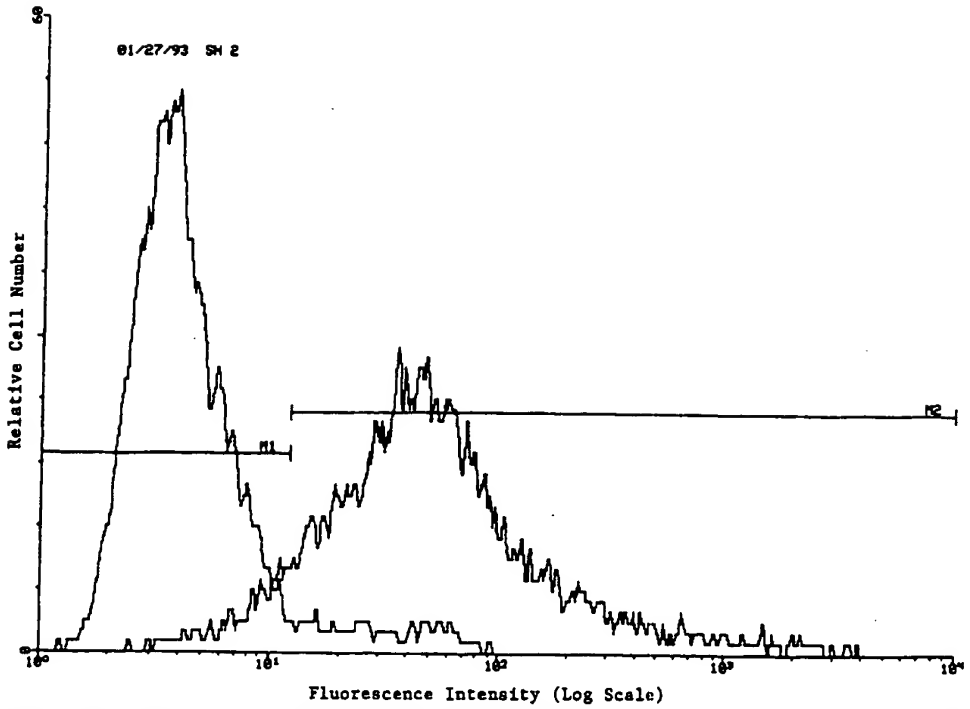

Fig. 5. Representative FACS analysis of mouse serum reactivity prior to (peak at 3) and after (peak at 50) immunization with G_{D3}-KLH and QS-21 tested on melanoma cell line SK-MEL-28.

used. When we attached G_{D3} to the amino terminal end of the MAP structure containing a malarial T-cell epitope, only a moderate IgM response against G_{D3} was detected, and there was no detectable IgG response. Conjugation of G_{D3} to polylysine resulted in a medium-titer IgM response and no IgG response, despite the high density of G_{D3} epitopes on these constructs.

The carrier that proved to be most effective in enhancing the antibody response to G_{D3} in this series was KLH. Immunization with G_{D3}-KLH consistently induced long-lasting production of IgM and IgG antibodies against G_{D3} at high titers. In comparing KLH with cBSA, OMP, MAP, and polylysine, it is difficult to know exactly why KLH is a superior carrier for G_{D3}. The sheer size and antigenic complexity of KLH stand out as a possible aid to antigen processing and recruitment of T-cell help across a broad range of T-cell specificities. The very qualities that make KLH cumbersome to work with are probably responsible for its unique effectiveness as a carrier in conjugate vaccines. KLH has not been widely used as a carrier for conjugate vaccines in humans because its size and heterogeneity make vaccine construction and standardization difficult.

Our hope was that conjugate vaccines would convert the T-cell-independent response against unconjugated G_{D3} seen in our previous studies to a T-cell-dependent response producing high-titer, long-lived, IgG antibodies. This expectation was fulfilled to some extent but not completely. The peak of the IgM response occurred after the third biweekly vaccination as in our previous studies with unconjugated G_{D3}, but the antibody titers were significantly higher. The response declined rapidly (as observed before), and additional vaccinations increased IgM titers to previous peak levels. The repeated increase in the titer of IgM antibodies to G_{D3} after booster immunizations differs from the expected response to T-cell-dependent antigens such as proteins, which generally induce little or no IgM response after booster immunizations. For the first time, however, we were able to induce a high-titer IgG response against G_{D3} ganglioside consistently. This response lasted significantly longer than the IgM response and was increased by additional vaccinations, although the response following booster vaccinations was not comparable to the exponential increase often seen with protein antigens. The fact that the G_{D3} antibodies were of the IgG1 subclass indicates that a T-cell-dependent pathway was activated by the G_{D3}-KLH conjugate vaccine. The lack of a classical booster effect, however, may reflect the carbohydrate nature of G_{D3} and its status as an auto-antigen. This suggests that T-cell recruitment by ganglioside conjugate vaccines is limited by the nature of the antigen itself. Nevertheless, the high-titer IgM response and long-lived IgG response to vaccination with G_{D3}-KLH and QS-21 seen in these experiments represents a striking improvement over the response to unconjugated ganglioside vaccines and can now form the basis for clinical trials of ganglioside-KLH conjugate vaccines in patients with cancers that show increased ganglioside expression.

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REFERENCES

1. Klenk, E. Z. Über die Ganglioside, eine neue Gruppe von zuckerhaltigen Gehirn Lipoiden. Physiol. Chem., *273:* 76–86, 1942.
2. Hakomori, S. I. Aberrant glycosylation in cancer cell membranes as focused on glycolipids: overview and perspectives. Cancer Res., *45:* 2405–2414, 1985.
3. Carubia, J. M., Yu, R. K., Mascala, L. J., Kirkwood, J. M., and Varga, J. M. Gangliosides on normal and neoplastic melanocytes. Biochem. Biophys. Res. Commun., *120:* 500–504, 1984.
4. Hamilton, W. B., Helling, F., Lloyd, K. O., and Livingston, P. O. Ganglioside expression on human malignant melanoma assessed by quantitative immune thin layer chromatography. Int. J. Cancer, *53:* 1–8, 1993.

5. Tsuchida, T., Saxton, R. E., Morton, D. L., and Irie, R. F. Gangliosides of human melanoma. J. Natl. Cancer Inst., *78l:* 45–54, 1987.
6. Houghton, A. N., Mintzer, D., Cordon-Cardo, C., Welt, S., Fliegel, B., Vadhan, S., Carswell, E., Melamed, M. R., Oettgen, H. F., and Old, L. J. Mouse monoclonal IgG3 antibody detecting G_{D3} ganglioside: a phase I trial in patients with malignant melanoma. Proc. Natl. Acad. Sci. USA, *82:* 1242–1246, 1985.
7. Cheung, N-K. V., Lazarus, H., Miraldi, F. D., Abramowsky, C. R., Kallic, S., Saarinen, U. M., Spitzer, T., Strandjord, S. E., Coccia, P. F., and Berger, N. A. Ganglioside G_{D2} specific monoclonal antibody 3F8: a phase I study in patients with neuroblastoma and malignant melanoma. J. Clin. Oncol., *5:* 1430–1440, 1987.
8. Irie, R. F., and Morton, D. L. Regression of cutaneous metastatic melanoma by intralesional injection with human monoclonal antibody to ganglioside G_{D2}. Proc. Natl. Acad. Sci. USA, *83:* 8694–8698, 1986.
9. Irie, R. F., Matsuki, T., and Morton, D. L. Human Monoclonal Antibody to Ganglioside G_{M2} for Melanoma Treatment. The Lancet, 786–787, 1989.
0. Livingston, P. O., Wong, G. Y., Adluri, S., Tao, Y., Padavan, M., Parente, R., Hanlon, C., Calves, M. J., Helling, F., Ritter, G., Oettgen, H. F., and Old, L. J. A randomized trial of adjuvant vaccination with BCG *versus* BCG plus the melanoma ganglioside G_{M2} in AJCC stage III melanoma patients. J. Clin. Oncol., in press, 1994.
1. Ritter, G., Boosfeld, E., Adluri, R., Calves, M. J., Oettgen, H. F., Old, L. J., and Livingston, P. O. Antibody response after immunization with ganglioside G_{D3} and G_{D3} congeners (lactones, amide and gangliosidol) in patients with malignant melanoma. Int. J. Cancer., *48:* 379–385, 1991.
2. Livingston, P. O., Natoli, E. J. Jr, Calves, M. J., Stockert, E., Oettgen, H. F., and Old, L. J. Vaccines containing purified G_{M2} ganglioside elicit G_{M2} antibodies in melanoma patients. Proc. Natl. Acad. Sci. USA, *84:* 2911–2915, 1987.
3. Livingston, P. O., Ritter, G., Srivastava, P., Padavan, M., Calves, M. J., Oettgen, H. F., and Old, L. J. Characterization of IgG and IgM antibodies induced in melanoma patients by immunization with purified G_{M2} ganglioside. Cancer Res., *49:* 7045–7050, 1989.
4. Eskola, J., Kayrty, H., Takala, A. K., Peltola, H., Ronneberg, P. R., Kha, E., Pekkanen, E., McVerry, P. H., and Makela, P. H. A randomized prospective field trial of a conjugate vaccine in the protection of infants and young children against invasive *Haemophilus influenzae* type b disease. N. Engl. J. Med., *323:* 1381–1387, 1990.
5. Anderson, P. Antibody response to *Haemophilus influenzae* type b and diphteria toxin induced by conjugates of oligosaccharides of the type b capsule with nontoxic protein CRM 197. Infect. Immun., *39:* 233–238, 1983.
6. Cahan, L. D., Irie, R. F., Singh, R., Cassidenti, A., and Paulson, J. C. Identification of a neuroectodermal tumor antigen (OFA-I-2) as ganglioside G_{D2}. Proc. Natl. Acad. Sci. USA, *79:* 7629–7633, 1982.
7. Ritter, G., Boosfeld, E., Calves, M. J., Oettgen, F. H., Old, L. J., and Livingston, P. O. Biochemical and serological characteristics of natural 9-*O*-acetyl G_{D3} from human melanoma and bovine buttermilk and chemically *O*-acetylated G_{D3}. Cancer Res., *50:* 1403–1410, 1990.
8. Ren, S., Scarsdale, J. N., Ariga, T., Zhang, Y., Klein, R. A., Hartmann, R., Kushi, Y., Egge, H., Yu, R. K. *O*-Acetylated gangliosides in bovine buttermilk. J. Biol. Chem., *267:* 12632–12638, 1992.
9. v. Nicolai, H., Müller, H. E., and Zilliken, F. Substrate specificity of neuraminidase from *Erysipelothrix rhusiopathiae*. Hoppe-Seyler's Z. Physiol. Chem., *359:* 393–398, 1978.
0. Kensil, C. R., Patel, U., Lennick, M., and Marciani, D. Separation and characterization of saponins with adjuvant activity from *Quillaja saponaria* molina cortex. J. Immunol., *146:* 431–437, 1991.
1. Dippold, W. G., Lloyd, K. O., Li, L. T., Ikeda, H., Oettgen, H. F., and Old, L. J. Cell surface antigens of human malignant melanoma: definition of six antigenic systems with monoclonal antibodies. Proc. Natl. Acad. Sci. USA, *77:* 6114–6118, 1980.
2. Criegee, R. The course of ozonization of unsaturated compounds. Rec. Chem. Prog., *18:* 111–120, 1957.
3. Wiegandt, H., and Baschang, G. Die Gewinnung des Zuckeranteils der Glykosphingolipide durch Ozonolyse und Fragmentierung. Z. Naturforsch., *20b:* 164–166, 1965.
4. Pappas, J. J., Keaveney, W. P., Gaucher, E., and Melvin, B. A new and convenient method for converting olefins to aldehydes. Tetrahedron Lett., *36:* 4273–4278, 1966.
5. Svennerholm, L. Quantitative estimation of sialic acids. II. Colorimetric resorcinol-hydrochloric acid method. Biochim. Biophys. Acta., *24:* 604–611, 1957.
6. Gray, G. R. The direct coupling of oligosaccharides to proteins and derivatised gels. Arch. Biochem. Biophys., *163:* 426–428, 1974.
7. Roy, R., and Lafferière, C. A. Michael addition as the key step in the synthesis of sialooligosaccharide protein conjugates from *N*-acroloylated glycopyranosyl-amines. J. Chem. Soc. Chem. Commun., 1709–1711, 1990.
8. Borch, R. F., Bernstein, M. D., and Durst, H. D. The cyanohydridoborate anion as a selective reducing agent. J. Am. Chem. Soc., *93:* 2897–2904, 1971.
9. Livingston, P. O., Koganty, R. R., Longenecker, B. M., Lloyd, K. O., and Calves, M. J. Studies on the immunogenicity of synthetic and natural Thomsen-Friedenreich (TF) antigens in mice: augmentation of the response by Quil A and SAF-m adjuvants and analysis of the specificity of the responses. Vaccine Res., *1:* 99–109, 1991.
0. Ritter, G., Boosfeld, E., Calves, M. J., Oettgen, H. F., Old, L. J., and Livingston, P. O. Antibody response after immunization with gangliosides G_{D3}, G_{D3} lactones, G_{D3} amide and G_{D3} gangliosidol in the mouse. G_{D3} lactone I indudes antibodies reactive with human melanoma. Immunobiology, *182:* 32–43, 1990.
1. Kanfer, J. N., and Hakomori, S. Sphingolipid biochemistry. *In:* D. J. Hanahan (ed.), Handbook of Lipid Research, Vol. 3, pp. 49–50. New York: Plenum Press, 1983.
2. Laine, R. A., Yogeeswaran, G., and Hakomori, S-I. Glycosphingolipids covalently linked to agarose gel or glass beads. J. Biol. Chem., *249:* 4460–4466, 1974.
3. Young, W. W., Jr., Laine, R. A., and Hakomori, S. An improved method for the covalent attachment of glycolipids to solid supports and macromolecules. J. Lipid. Res., *20:* 275–278, 1979.
4. Lowell, G. H., Ballou, W. R., Smith, L. F., Wirtz, R. A., Zollinger, W. D., and Hockmeyer, W. T. Proteosome-lipopeptide vaccines: enhancement of immunogenicity for malaria CS peptides. Science (Washington DC), *240:* 800–802, 1988.
5. Donnelly, J. J., Deck, R. R., and Liu, M. A. Adjuvant activity of the outer membrane protein complex of Neisseria meningitidis serogroup B for a polysaccharide-protein conjugate. Vaccines, *91:* 403–408, 1991.
6. Livingston, P. O., Calves, M. J., Helling, F., Zollinger, W. O., Blake, M. S., and Lowell, G. H. G_{D3}/proteosomes vaccine induce consistent IgM antibodies against the ganglioside G_{D3}. Vaccine, *11:* 1199–1204, 1993.
7. Apple, R. J., Domen, P. L., Muckerheide, A., and Michael, J. G. Cationization of protein antigens IV: increased antigen uptake by antigen presenting cells. J. Immunol., *40:* 3290–3295, 1988.
8. Tam, J. P. Synthetic peptide vaccine design: synthesis and properties of a high-density multiple antigenic peptide system. Proc. Natl. Acad. Sci. USA, *85:* 5409–5413, 1988.
9. Tam, J. P., and Lu, Y. Vaccine engineering: Enhancement of immunogenicity of synthetic peptide vaccines related to hepatitis in chemically defined models consisting of T- and B-cell epitopes. Proc. Natl. Acad. Sci. USA, *86:* 9084–9088, 1989.
0. Svennerholm, L. Chromatographic separation of human brain gangliosides. J. Neurochem., *10:* 613–623, 1963.

G_{D3} Vaccines for Melanoma: Superior Immunogenicity of Keyhole Limpet Hemocyanin Conjugate Vaccines[1]

Friedhelm Helling,[2] Ann Shang, Michele Calves, Shengle Zhang, Shunlin Ren, Robert K. Yu, Herbert F. Oettgen, and Philip O. Livingston

Immunology Program and Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, New York 10021 [F. H., A. S., M. C., S. Z., H. F. O., P. O. L.], and Department of Biochemistry and Molecular Biophysics, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298-0614 [S. R., R. K. Y.]

ABSTRACT

Cell surface gangliosides show altered patterns of expression as a consequence of malignant transformation and have therefore been of interest as potential targets for immunotherapy, including vaccine construction. One obstacle has been that some of the gangliosides that are overexpressed in human cancers are poorly immunogenic in humans. A case in point is G_{D3}, a prominent ganglioside of human malignant melanoma. Using an approach that has been effective in the construction of bacterial carbohydrate vaccines, we have succeeded in increasing the immunogenicity of G_{D3} in the mouse by conjugating the ganglioside with immunogenic carriers. Several conjugation methods were used. The optimal procedure involved ozone cleavage of the double bond of G_{D3} in the ceramide backbone, introducing an aldehyde group, and coupling to aminolysyl groups of proteins by reductive amination. Conjugates were constructed with a synthetic multiple antigenic peptide expressing repeats of a malarial T-cell epitope, outer membrane proteins of *Neisseria meningitidis*, cationized bovine serum albumin, keyhole limpet hemocyanin, and polylysine. Mice immunized with these conjugates showed a stronger antibody response to G_{D3} than mice immunized with unconjugated G_{D3}. The strongest response was observed in mice immunized with the keyhole limpet hemocyanin conjugate of the G_{D3} aldehyde derivative and the adjuvant QS-21. These mice showed not only a long-lasting high-titer IgM response but also a consistent high-titer IgG response (predominantly IgG1), indicating recruitment of T-cell help, although the titers of IgM and IgG antibodies following booster immunizations were not as high as they are in the response to classical T-cell-dependent antigens. This method is applicable to other gangliosides, and it may be useful in the construction of immunogenic ganglioside vaccines for the immunotherapy of human cancers expressing gangliosides on their cell surface.

INTRODUCTION

Gangliosides are glycolipid constituents of the cell membrane. The term was coined in 1942 to refer to lipids of the central nervous system that contained sialic acid, to signify their prime location in ganglion cells and their glycosidic nature (1). Their lipophilic component, the ceramide (an amide-linked long-chain sphingoid base and a fatty acid), is thought to be embedded in the outer membrane of the cell membrane lipid bilayer. The carbohydrate portion of the molecule is oriented toward the outside of the cell. Malignant transformation appears to activate enzymes involved in ganglioside glycosylation, resulting in altered patterns of ganglioside expression in tumors such as astrocytoma, neuroblastoma, and malignant melanoma (2). In normal melanocytes, for example, the predominant ganglioside is G_{M3}.[3] Other gangliosides including G_{D3}, G_{M2}, G_{D1a}, and G_{T1b} constitute less than 10% of the total (3). In malignant melanoma, increased expression of G_{D3}, G_{D2}, and G_{M2} has been observed (4, 5), and these gangliosides have therefore been considered potential targets for immunotherapy.

One approach to ganglioside-targeted immunotherapy has been the use of mAbs.[4] Treatment of patients with melanoma or neuroblastoma with mAb recognizing G_{D3}, G_{D2}, or G_{M2} has resulted in tumor regression in some cases (6–9). The other approach has been to immunize patients with ganglioside vaccines in attempts to induce production of ganglioside antibodies by the patients themselves. These attempts have been successful so far only with G_{M2} vaccines. Patients with American Joint Committee on Cancer Stage III malignant melanoma, after complete resection of all tumor, have been shown to produce anti-G_{M2} antibodies in response to vaccination with G_{M2} and *Bacillus Calmette-Guérin* (after pretreatment with low-dose cyclophosphamide to reduce suppressor activity), and the disease-free interval and overall survival were longer in patients producing G_{M2} antibodies (10). G_{D3} and G_{D2}, on the other hand, were found to be only rarely immunogenic when administered in the same way to patients with melanoma (11). Even with the G_{M2} vaccines, the antibody response showed the characteristics of a T-cell-independent response, that is to say, IgM production of short duration, rare conversion to IgG production, and lack of a booster effect (12, 13).

Similar difficulties have been encountered in the development of effective vaccines against bacterial carbohydrate antigens. One approach that has been successful in overcoming these problems is conjugation of the antigen with immunogenic protein carriers. For example, a conjugate vaccine that links the *Haemophilus influenzae* type b capsular polysaccharide to the outer-membrane protein complex of *Neisseria meningitidis* serogroup B was recently shown to induce the production of antibodies and a high rate of protection against invasive disease caused by *Haemophilus influenzae* type b in infants (14), and similar results were reported for a conjugate vaccine using a nontoxic mutant diphtheria toxin as carrier (15).

We have explored this approach in attempts to increase the immunogenicity of melanoma gangliosides. We report here the effects of conjugating G_{D3} with several protein carriers on its immunogenicity in the mouse.

MATERIALS AND METHODS

Gangliosides. G_{M3}, G_{M2} and G_{D1b}, extracted from bovine brain, were provided by Fidia Research Laboratory (Abano Terme, Italy). G_{D2} was made from G_{D1b} by enzymatic cleavage with β-galactosidase from bovine testes (16). G_{D3} (mel) was isolated from human melanoma tissue (17), G_{D3} (bbm) and GT3 were isolated from bovine buttermilk (18), and disialyllactose (G_{D3} oligosaccharide) was isolated from bovine colostrum as previously described (19).

Reagents. HPTLC silica gel plates were obtained from E. Merck (Darmstadt, Germany); 4-chloro-1-naphthol, *p*-nitrophenyl phosphate disodium, and sodium cyanoborohydride were from Sigma Chemical Co. (St. Louis, MO);

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[2] To whom requests for reprints should be addressed, at Immunology Program, Memorial Sloan-Kettering Cancer Center, 425 East 68th Street, Room 820 K, New York, NY 10021.

[3] The designations G_{M3}, G_{M2}, G_{M1}, G_{D3}, G_{D1a}, and G_{D1b} are used in accordance with the abreviated ganglioside nomenclature proposed by Svennerholm (40).

[4] The abbreviations used are: mAb, monoclonal antibody; MAP, multiple antigenic peptide; OMP, outer membrane protein; cBSA, cationized bovine serum albumin; ITLC, immune thin-layer chromatography; HPTLC, high-performance thin-layer chromatography; ELISA, enzyme-linked immunosorbent assays; FACS, fluorescence-activated cell sorter; PBS, phosphate-buffered saline; bbm, bovine buttermilk.

methylsulfide was from Aldrich (Milwaukee, WI); cyclophosphamide (Cytoxan) was from Mead Johnson (Syracuse, NY); and QS-21 adjuvant, a homogeneous saponin component purified from *Quillaja saponaria* Molina tree (20), was kindly donated by Cambridge Biotech Corp. (Worcester, MA). It is an amphipathic molecule and was provided as a white powder, forming a clear colorless solution when dissolved in PBS.

Proteins. Poly-L-lysine hydrobromide [MW(vis)3800] was purchased from Sigma, keyhole limpet hemocyanin (KLH) was from Calbiochem (La Jolla, CA), the cBSA-Imject Supercarrier immune modulator was from Pierce (Rockfort, IL), and *Neisseria meningitidis* OMPs were kindly provided by Dr. M. S. Blake (Rockefeller University, New York). MAP YAL-IV 294-I containing four repeats of a malarial T-cell epitope was a gift from Dr. J. P. Tam (Rockefeller University).

Monoclonal Antibodies. Rabbit anti-mouse immunoglobulins conjugated to horseradish peroxidase for ITLC, and rabbit anti-mouse IgM and IgG conjugated to alkaline phosphatase for ELISAs, were obtained from Zymed (San Francisco, CA); anti-G_{D3} mAb R24 was generated in our laboratory (21).

Serological Assays. ELISA were performed as previously described (13). To control for nonspecific "stickiness," immune sera were also tested on plates to which no ganglioside had been added, and the reading was subtracted from the value obtained in the presence of ganglioside. The titer was defined as the highest dilution yielding a corrected absorbance of 0.1 or greater. Immunostaining of gangliosides with mAb or mouse sera was performed after separation on HPTLC silica gel glass plates as previously described (4). Plates were developed in solvent 1 [chloroform:methanol:water (0.25% $CaCl_2$), 50:40:10 (v/v)] or solvent 2 [ethanol:*n*-butylalcohol:pyridine:water:acetic acid, 100:10:10:30:3 (v/v)], and gangliosides were visualized with resorcinol-HCl reagent. Dot-blot immune stains were performed on nitrocellulose strips utilizing purified gangliosides spotted in equal amounts and developed as described before (13).

Immunization. Six-week-old female BALB/c × C57BL/6 F_1 mice (The Jackson Laboratory, Bar Harbor, ME) were given an i.p. injection of cyclophosphamide (15 mg/kg) 3 days before the first immunization and were then assigned to treatment groups. Groups of 4 or 5 mice were given three s.c. injections of a vaccine 2 weeks apart if not otherwise indicated. Each vaccine contained 20 μg G_{D3} or 15 μg disialyllactose and 10 μg QS-21 in a total volume of 0.1 ml PBS. Mice were bled from the retroorbital sinus before vaccination and 2 weeks after the last vaccine injection unless indicated otherwise.

G_{D3} Conjugate Preparation. G_{D3} (2 mg) was dissolved in 2 ml methanol by sonication and cooled to −78°C in an ethanol/dry ice bath. Ozone was generated in an ozone generator (Del Industries, San Luis Obispo, CA) and was passed through the sample for 30 min under vigorous stirring (22, 23). The excess of ozone was then displaced with nitrogen over a period of 10 min. Methylsulfide (100 μl) was added (24), and the sample was kept at −78°C for 30 min and then at room temperature for 90 min under vigorous stirring. The sample was dried under a stream of nitrogen and monitored by HPTLC. The long-chain aldehyde was separated by adding *n*-hexane (2 ml) to the dry sample, followed by sonication for 5 min and centrifugation at 2000 × *g* for 15 min. The *n*-hexane was carefully drawn off and discarded, and the sample was dried under a stream of nitrogen. Cleaved G_{D3} and native G_{D3} were separated by HPLC (Waters, System 501, Milford, MA) utilizing a C_{18} reversed-phase column (10 x 250 mm; Rainin Instruments, Ridgefield, NJ). Gangliosides were eluted with a linear water-acetonitrile gradient and monitored at 214 nm, and the fractions were analyzed by HPTLC. Fractions that contained cleaved G_{D3} were combined and evaporated at 37°C with a Rotavapor (Büchi, Flawils, Switzerland). Cleaved G_{D3} (1.5 mg), 1.5 mg protein carrier in PBS, and 2 mg sodium cyanoborohydride were incubated under gentle agitation at 37°C for 48 h. After 16 h 1 mg sodium cyanoborohydride was added. The progress of coupling was monitored by HPTLC. G_{D3}-protein conjugates did not migrate in solvent 1 and solvent 2 but remained at the origin as a resorcinol-positive band. The mixture was dialyzed across 5000 molecular weight cutoff dialysis tubing with three changes of PBS (4 liters each), at 4°C for 48 h, and passed through an Extractigel detergent-removing gel (Pierce, Rockfort, IL) for final purification of unconjugated G_{D3}. The samples were lyophilized, and their protein and ganglioside content was determined by BioRad protein assay and by neuraminic acid determination according to the method of Svennerholm (25).

Disialyllactose Conjugate Preparation. Disialyllactose was isolated from bovine colostrum as described previously (19). The carbohydrate was attached to protein by reductive amination (26). Disialyllactose (10 mg) was incubated with 2 mg proteins in 2 ml PBS for 14 days at 37°C after sterile filtration. Sodium cyanoborohydride (2 mg) was added at the beginning, and 1 mg was added every 3 days. The coupling was monitored by HPTLC in solvent 2. The disialyllactose conjugates were purified by dialysis across 5000 molecular weight cutoff dialysis membrane with three changes of PBS (4 liters each) at 4°C for 48 h, followed by lyophilization. The protein and neuraminic acid content was determined as described above. Disialyllactose was also conjugated to proteins according to the method described by Roy and Laferrière (27). During this procedure *N*-acroloylated glycopyranosylamine derivatives of the oligosaccharide were formed first, followed by conjugation via Michael addition to amino groups of the protein. Purification and determination of protein and neuraminic acid content were performed as described above.

Determination of Antibody Subclasses. Determination of antibody subclasses was performed by ELISA using subclass-specific rabbit anti-mouse immunoglobulins IgG1, IgG2a, IgG2b, IgG3, and IgA (Zymed, San Francisco, CA). Alkaline phosphatase-labeled goat anti-rabbit IgG served as the signal-generating reagent.

FACS Analysis of Mouse Antisera. A single cell suspension of the melanoma cell line SK-MEL-28 was obtained after treatment with 0.1% EDTA in PBS followed by passage through a 26½-gauge needle. Cells (3×10^5) were incubated with 40 μl of 1:20 diluted post- or preimmunization serum for 30 min on ice. The cells were washed three times with 3% fetal calf serum in PBS. Thirty μl of diluted (1:50) fluorescein isothiocyanate-labeled goat anti-mouse IgG (Southern Biotechnology Associates Inc., Birmingham, AL) were added as secondary antibody, followed by incubation on ice for 30 min. Cells were washed three times as above and resuspended in 500 μl 3% fetal calf serum in PBS and analyzed by flow cytometry (FACScan, Becton Dickinson, San Jose, CA).

RESULTS

Preparation and Characterization of G_{D3}-Protein Conjugates. G_{D3} (bbm) in methanol was selectively cleaved with ozone at the C4-C5 double bond in the ceramide portion. It is assumed that methoxyperoxides are formed as intermediate products (24), and therefore methylsulfide was added as a reducing agent. The result of the cleavage was a G_{D3} derivative with an aldehyde functional group in the position of the former double bond in the ceramide portion (Fig. 1). Cleaved G_{D3} migrated slower than native G_{D3}, and formed double bands because the ceramide contained unsaturated fatty acids that were cleaved simultaneously (see Fig. 1, *inset*). Densitometric analysis of HPTLC plates showed that more than 70% of G_{D3} (bbm) was cleaved by this procedure. Preliminary experiments involving longer ozone treatment had similar results, indicating that 30% of G_{D3} from this source consists of sphinganine or phytosphingosine analogues that contain no ozone-cleavable ceramide double bond. Cleavage at −78°C with ozone treatment up to 1 h (depending on the amount of G_{D3} used) was found to be optimal. Cleaved G_{D3} persisted only in acidic and neutral phosphate buffers for up to 72 h, but with the formation of increasing amounts of oligosaccharide due to β-elimination reactions [which have been shown to occur much faster at alkaline pH (23)]. The decreased hydrophobicity of cleaved G_{D3} compared to native G_{D3} allowed its separation by HPLC on C_{18} reversed-phase columns. Utilizing isocratic elution with a linear water-acetonitrile gradient, cleaved G_{D3} was recovered first, and uncleaved G_{D3} was eluted in later fractions. The incubation of cleaved G_{D3} with proteins resulted in the formation of Schiff bases between the cleaved ganglioside and ε-aminolysyl groups. They were reduced with sodium cyanoborohydride to form stable secondary amine bonds (28). The reaction was monitored by HPTLC, which showed a decreasing ratio of the cleaved G_{D3} to a resorcinol positive band at the origin, indicating the formation of neoglycoconjugates. The reaction was generally completed after incubation for 48 h at 37°C. Disialyllactose was readily remov-

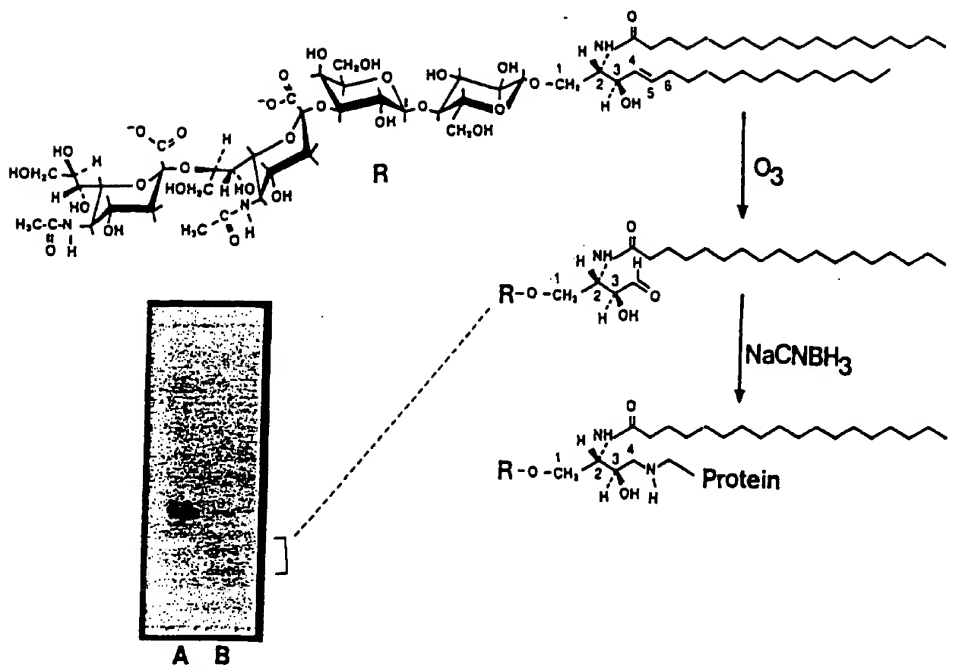

Fig. 1. Synthesis of G_{D3} protein conjugates after ozone cleavage and reductive amination. *Inset*, HPTLC of G_{D3} before (*A*) and after (*B*) ozone cleavage.

ible by dialysis, and the excess of cleaved G_{D3} was removed by passage through a detergent-removing column. The degree of coupling was determined by sialic acid and protein determinations. The weight ratio of G_{D3} to proteins in the different conjugates, shown in Table 1, depended on the accessibility of lysine groups in the proteins. The average yield of G_{D3} coupled to proteins was 30%. G_{D3} conjugates prepared in this way were reactive with anti-G_{D3} mAb R24 by Western blot analysis, although the G_{D3}-aldehyde derivative itself was not reactive by ITLC (data not shown).

Oligosaccharide Conjugation. The carbohydrate part of G_{D3}, disialyllactose, was coupled to proteins utilizing two methods. The first method, reductive amination, resulted in conjugation of the open ring form of the glucose to proteins (26). The method required a long incubation of the oligosaccharide with proteins, and the yield was less than 20%. In the second method (27), involving *N*-acroloylation of the terminal glucose, the oligosaccharide was coupled to proteins with a closed ring formation. None of these oligosaccharide conjugates showed reactivity with mAb R24 by Western blot analysis (data not shown).

Induction of a Serological Response against G_{D3} by Immunization with G_{D3}-Protein Conjugates. All vaccines were well tolerated. Mice were observed for at least 6 months, and neither acute nor systemic toxicity was detected. The serological response to immunization with G_{D3} or G_{D3}-protein conjugates, using QS-21 as adjuvant, is shown in Table 1. QS-21 was used because we had previously demonstrated its superiority over other adjuvants with another carbohydrate antigen-KLH conjugate vaccine (29). In ELISA, preimmunization sera showed no IgM or IgG antibodies reactive with G_{D3}. Immunization with unconjugated G_{D3} did not induce the production of G_{D3} antibodies. Immunization with G_{D3} conjugates, on the other hand, was effective in inducing antibody production. Of the five proteins used in the preparation of the conjugates, KLH showed the

Table 1 *Antibody response to immunization with different vaccines containing G_{D3} or disialyllactose conjugated to carrier proteins*

Vaccine + QS-21	No. of mice	G_{D3}:protein weight ratio[a]	Reciprocal ELISA peak titer against G_{D3}	
			IgG	IgM
G_{D3}	5		0 (5)	20 (3), 0 (2)
G_{D3}/KLH[b]	5	0.33	0 (5)	160, 40, 20 (3)
G_{D3}-KLH[c]	14	0.69	10,240 (2), 5,120 (2), 2,560 (3), 1,280 (2), 80 (2), 40 (2), 0	2,560, 1,280 (2), 640, 320 (3), 160 (2), 80 (3), 20, 0
G_{D3}-cBSA[c]	15	0.77	2,560 (2), 320 (2), 160, 80 (2), 40 (4), 20 (2), 0 (2)	80 (2), 40 (2), 20 (7), 0 (4)
G_{D3}-OMP[c]	15	0.93	2,560, 80 (4), 20 (3), 0 (7)	1,280, 320 (2), 160 (7), 80 (4), 40
G_{D3}-MAP[c]	10	1.0	40, 0 (0)	160 (2), 40 (4), 20 (3), 0
G_{D3}-Polylysine	10	ND	0 (10)	320, 160 (4), 80, 40, 20 (2), 0
Disialyllactose-KLH[d]	4	0.055	0 (4)	160 (3), 80
Disialyllactose-cBSA[d]	4	0.16	20, 0 (3)	40, 20 (3)
Disialyllactose-KLH[e]	4	0.25	20, 0 (3)	40 (2), 0 (2)
Disialyllactose-cBSA[e]	4	0.34	0 (4)	0 (4)
Disialyllactose-Polylysine	5	ND	0 (5)	80 (3), 40 (2)

[a] Protein and ganglioside content were determined by BioRad protein assay and by neuraminic acid determination according to the method of Svennerholm (25).
[b] G_{D3} and KLH were mixed prior to immunization.
[c] G_{D3} was covalently attached to proteins prior to immunization after ozonolysis as described in "Materials and Methods."
[d] Disiallylactose was conjugated to KLH and cBSA by reductive amination according to the method of Gray (26).
[e] Disiallylactose was conjugated to KLH, cBSA, and poly-L-lysine after *N*-acroloylation and Michael addition according to the method of Roy and Laferière (27).

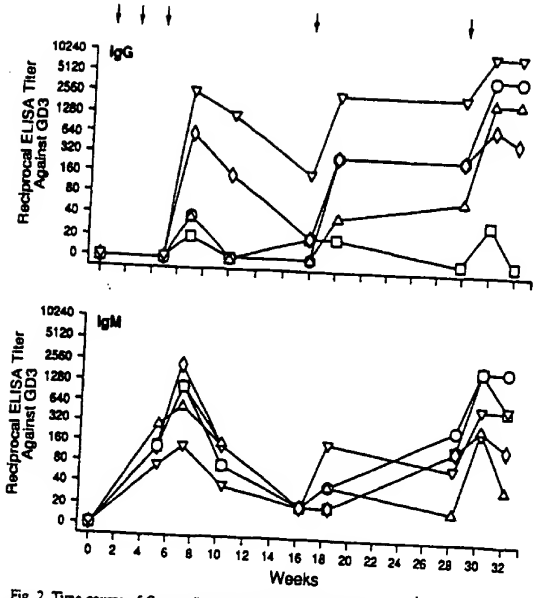

Fig. 2. Time course of G_{D3} antibodies induced in representative mice immunized with G_{D3}-KLH and QS-21 vaccine. Each symbol represents an individual mouse. Arrows, time of vaccination.

strongest immunogenicity, resulting in a median titer of 1:320 for IgM and 1:2560 for IgG antibodies. The specific isotype profile was determined with subclass-specific secondary rabbit anti-mouse antibodies. Antigen-specific antibodies were found to be predominantly of the IgG1 subclass. Antigen-specific IgG2a and IgG2b antibodies were found only in traces, and no IgG3 or IgA antibodies were detected.

In contrast to immunization with G_{D3} conjugates, immunization with G_{D3}-oligosaccharide conjugates induced only a weak IgM response to G_{D3} and no IgG response.

Sequential IgM and IgG antibody titers against G_{D3} for five mice immunized with G_{D3}-KLH and QS-21 are shown in Fig. 2. IgM titers peaked 2 weeks after the third vaccination and declined by the time of the first booster immunization at week 16. The first booster immunization had no significant impact on IgM titers, but the second booster immunization at week 28 increased IgM titers to the peak level seen after the third vaccination of the initial series. IgG titers also rose up to 2 weeks after the third vaccination and decreased by the time of the first booster vaccination but rapidly increased after the booster to previous peak titers. IgG titers remained at this level for 10 weeks, with a further increase after the second booster in most mice. The evidence for a secondary immune response after the booster immunization was therefore equivocal. The response was clearly more rapid than after the initial immunization and lasted longer, but the increase in titer was not comparable to booster responses seen with classical T-cell-dependent antigens.

Specificity of the Serological Response to Immunization with G_{D3}-Protein Conjugates. The specificity of the serological response to immunization with G_{D3}-protein conjugates and QS-21 was analyzed by dot-blot immune staining and ITLC. An example of dot-blot immune stain analysis is shown in Fig. 3. Preimmune sera and immune sera showing high G_{D3}-antibody titers in ELISA were tested on nitrocellulose strips that had been spotted with G_{D3} (bbm) or G_{D3} (mel) and purified structurally related gangliosides: G_{M3}, G_{D2}, G_{D1b}, and G_{T3}. As expected on the basis of the ELISA results, preimmune sera showed no reactivity. In contrast, sera obtained after immunization with KLH conjugates of G_{D3}-ganglioside reacted with G_{D3} (bbm) (the immunogen) or G_{D3} (mel), but not with the other gangliosides except G_{T3} in some cases, a pattern also seen in tests of the mouse monoclonal IgG3 antibody R24, the reagent by which high cell surface expression of G_{D3} on human melanoma cells was first defined (20). The same specificity pattern was seen in dot-blot immune stain tests of sera from mice immunized with other G_{D3}-protein conjugates, the only exception being high-titer sera (by ELISA) from mice immunized with G_{D3}-cBSA, which showed no reactivity with G_{D3} or the other gangliosides.

ITLC permits specificity analysis of ganglioside antibodies in tests on tissue extracts. Examples of tests with high-titer sera from mice immunized with G_{D3}-KLH and QS-21 are shown in Fig. 4. The sera were tested at a dilution of 1:150 on ganglioside extracts of human brain, neuroblastoma, and melanoma, as well as G_{D3} (bbm) that had been used for immunization. The figure shows HPTLC ganglioside patterns of these reagents after staining with resorcinol, as compared with the patterns of reactivity exhibited after exposure to sera from immunized mice or mAb R24. As can be seen in the resorcinol-stained panel, the predominant gangliosides in the brain tissue extract are G_{M1}, G_{D1a}, G_{D1b}, and G_{T1b}, whereas the neuroblastoma extract shows G_{D2} and G_{M2} in addition, and the melanoma extract contains mainly

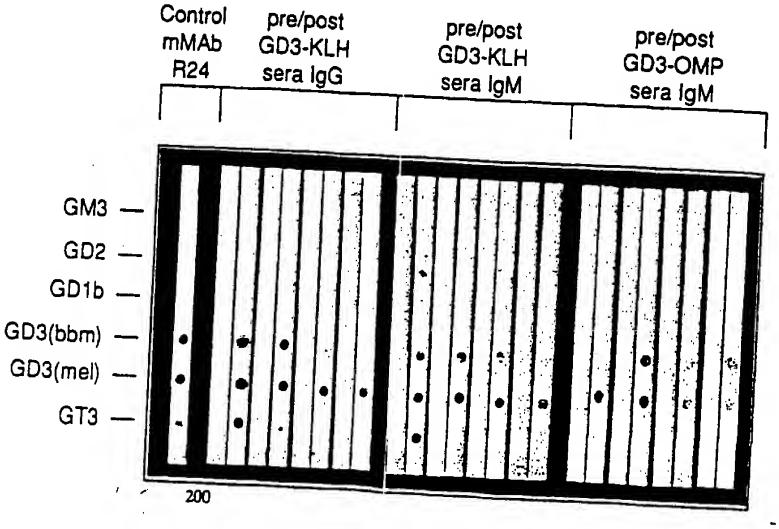

Fig. 3. Dot-blot immune stain assay for IgM and IgG antibodies in sera of mice immunized with G_{D3}-KLH and G_{D3}-OMP conjugates and QS-21. Antigen standards were applied to nitrocellulose strips in equal amounts (0.5 μg) and were allowed to react with pre/postimmunization serum from individual mice.

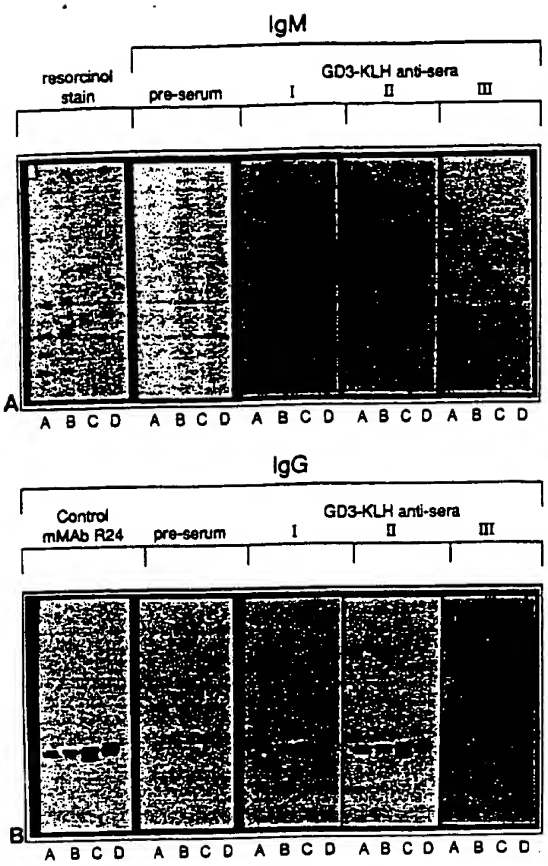

Fig. 4. Immune thin-layer chromatograms of three representative mouse sera after accination with G_{D3}-KLH conjugate and QS-21. IgG and IgM antibodies in pre- and ıstvaccination sera and anti-G_{D3} mMab R24 were tested on human brain gangliosides), neuroblastoma gangliosides (*B*), melanoma gangliosides (*C*), and G_{D3} (*D*) (bbm). angliosides were chemically stained with resorcinol-HCl reagent to demonstrate the ıngliosides composition of each sample.

$_{D3}$ and G_{M3}. Reactivity of IgG antibodies in postimmunization sera, ; well the reactivity of IgG3 mouse monoclonal antibody R24, was :stricted to G_{D3} (Fig. 4*b*). The high-titer IgM antibodies, on the other and, showed weak cross-reactivity with other gangliosides and sul- ıtide in the brain extract (Fig. 4*a*).

Sera from mice immunized with other G_{D3} conjugates were tested ı the same way (at lower dilution) and showed the same specificity ·ith the exception, again, of high-titer sera from mice immunized ·ith G_{D3}-cBSA, which showed no ganglioside reactivity (data not ıown).

Cell Surface Reactivity of Immune Sera Determined by FACS ıalysis. Sera from mice were tested for binding to cells of the ıelanoma cell line SK-MEL-28, a cell line known to express cell ırface G_{D3}. A representative example of a FACS analysis utilizing a uorescein isothiocyanate-labeled secondary goat anti-mouse anti- ody is shown in Fig. 5. Sera before and after immunization with $_{D3}$-KLH and QS-21 were tested. Preimmunization serum stained 8% f the target cells, postimmunization serum 92%.

ıISCUSSION

Conjugation of poorly immunogenic antigens to highly immuno- enic carrier molecules is a well-known approach to augmenting nmunogenicity. Ganglioside molecules are so small, however, that linkage to carrier molecules without affecting the relevant antigenic epitopes is difficult. We have shown previously that modifications of G_{D3} in its carbohydrate portion (*i.e.*, conversion of sialic acid carboxyl groups to amides or gangliosidols or lactones) results in markedly increased immunogenicity. However, antibodies produced in response to these G_{D3} derivatives show no cross-reactivity with native G_{D3} (11, 30). Covalent attachment of proteins to the sialic acid molecules of G_{D3} was therefore not attempted in the present study. Our initial approach involved conjugation of G_{D3} oligosaccharide (disialyllactose) via the terminal glucose in open- or closed-ring configuration to KLH or polylysine, but these conjugates were not recognized by the anti-G_{D3} mAb R24 or by mouse antisera to G_{D3}, and mice immunized with the conjugates did not produce G_{D3} antibodies. Subsequently, we coupled G_{D3} to proteins via its ceramide portion without alteration of the carbohydrate moiety. The ceramide was cleaved with ozone at the double bond of the sphingosin base, and coupling to proteins was accomplished by reductive amination. Cleavage of gangliosides by ozonolysis and subsequent conjugation with proteins by this method has not been described, and it has been generally assumed that the aldehyde intermediates of gangliosides would be unstable. Fragmentation, initiated by hydroxy ions under alkaline conditions, has been reported. Migration of the double bond would result in β-elimination, causing release of the oligosaccharide moiety (22, 31). We found, however, that the aldehyde was sufficiently stable at neutral pH to permit Schiff base formation with amino groups of proteins, so that β-elimination was not a major problem. The overall yield was 30%. These G_{D3} aldehyde-protein conjugates showed reactivity with G_{D3} antibodies by Western blot analysis, indicating that the immunodominant epitopes were intact in these G_{D3} conjugates. However, reactivity of the G_{D3}-aldehyde derivative with mAb R24 by ITLC could not be shown. This may be due to its relatively unstable nature, resulting in β-elimination and release of oligosaccharide during the immune stain incubation period, or simply to the fact that the G_{D3}-aldehyde derivative may not adhere to the thin-layer plate sufficiently for serological detection.

Earlier studies describe oxidative ozonolysis of the glycosphingolipid olefinic bond, resulting in a carboxyl group that could be conjugated with carbodiimide to NH_2 groups of modified glass beads, agarose gel, or other macromolecules (32, 33). This method, however, is of limited use for the conjugation of gangliosides to carrier proteins because it requires acetylated, methyl ester derivatives of gangliosides to avoid coupling via the sialic acid carboxyl group. Deacetylation after conjugation under basic conditions is necessary, conditions most proteins cannot be exposed to without degradation.

Once the conjugation method was established, several protein carriers were considered, based on previous work by others. Lowell *et al.* (34) described an elegant system that resulted in high-titer antibody responses as a consequence of anchoring bacterial carbohydrate and peptide antigens via a synthetic, hydrophobic foot in OMPs of *Neisseria meningitidis* (35). This system was directly applicable to gangliosides because of their amphipathic nature. In previous studies, we adsorbed gangliosides onto OMP by hydrophobic interaction, and we were able to induce high-titer IgM responses (36). Covalent attachment was utilized in the current study, but G_{D3}-OMP conjugates induced only occasional IgG responses, and the IgM response was not increased. Conjugation with cationized BSA, which has been reported to be a potent carrier for protein antigens (37), resulted in high-titer IgG antibodies detected by ELISA, but immune stains indicated that the response was not G_{D3}-specific. Another appealing carrier is the MAP system described by J. P. Tam (38, 39). MAPs consist of four or eight dendritic peptide arms, containing B- and T-cell epitopes, attached to an oligomeric branched lysine core. The antibody response to peptides was dramatically increased when these constructs were

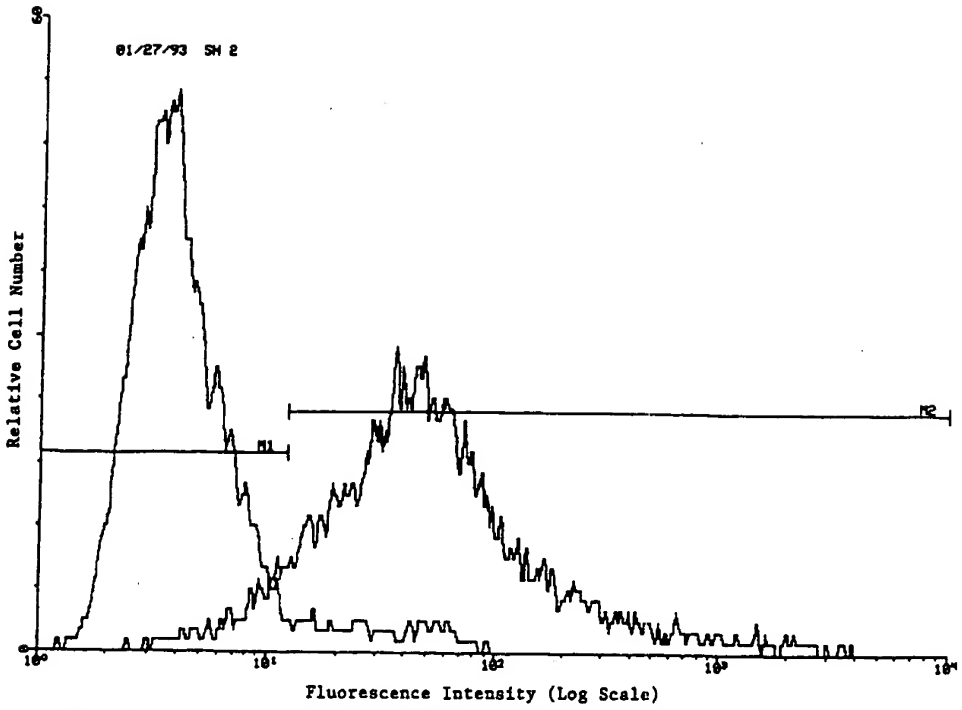

Fig. 5. Representative FACS analysis of mouse serum reactivity prior to (peak at 3) and after (peak at 50) immunization with G_{D3}-KLH and QS-21 tested on melanoma cell line SK-MEL-28.

used. When we attached G_{D3} to the amino terminal end of the MAP structure containing a malarial T-cell epitope, only a moderate IgM response against G_{D3} was detected, and there was no detectable IgG response. Conjugation of G_{D3} to polylysine resulted in a medium-titer IgM response and no IgG response, despite the high density of G_{D3} epitopes on these constructs.

The carrier that proved to be most effective in enhancing the antibody response to G_{D3} in this series was KLH. Immunization with G_{D3}-KLH consistently induced long-lasting production of IgM and IgG antibodies against G_{D3} at high titers. In comparing KLH with cBSA, OMP, MAP, and polylysine, it is difficult to know exactly why KLH is a superior carrier for G_{D3}. The sheer size and antigenic complexity of KLH stand out as a possible aid to antigen processing and recruitment of T-cell help across a broad range of T-cell specificities. The very qualities that make KLH cumbersome to work with are probably responsible for its unique effectiveness as a carrier in conjugate vaccines. KLH has not been widely used as a carrier for conjugate vaccines in humans because its size and heterogeneity make vaccine construction and standardization difficult.

Our hope was that conjugate vaccines would convert the T-cell-independent response against unconjugated G_{D3} seen in our previous studies to a T-cell-dependent response producing high-titer, long-lived, IgG antibodies. This expectation was fulfilled to some extent but not completely. The peak of the IgM response occurred after the third biweekly vaccination as in our previous studies with unconjugated G_{D3}, but the antibody titers were significantly higher. The response declined rapidly (as observed before), and additional vaccinations increased IgM titers to previous peak levels. The repeated increase in the titer of IgM antibodies to G_{D3} after booster immunizations differs from the expected response to T-cell-dependent antigens such as proteins, which generally induce little or no IgM response after booster immunizations. For the first time, however, we were able to induce a high-titer IgG response against G_{D3} ganglioside consistently. This response lasted significantly longer than the IgM response and was increased by additional vaccinations, although the response following booster vaccinations was not comparable to the exponential increase often seen with protein antigens. The fact that the G_{D3} antibodies were of the IgG1 subclass indicates that a T-cell-dependent pathway was activated by the G_{D3}-KLH conjugate vaccine. The lack of a classical booster effect, however, may reflect the carbohydrate nature of G_{D3} and its status as an auto-antigen. This suggests that T-cell recruitment by ganglioside conjugate vaccines is limited by the nature of the antigen itself. Nevertheless, the high-titer IgM response and long-lived IgG response to vaccination with G_{D3}-KLH and QS-21 seen in these experiments represents a striking improvement over the response to unconjugated ganglioside vaccines and can now form the basis for clinical trials of ganglioside-KLH conjugate vaccines in patients with cancers that show increased ganglioside expression.

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REFERENCES

1. Klenk, E. Z. Über die Ganglioside, eine neue Gruppe von zuckerhaltigen Gehirn Lipoiden. Physiol. Chem., *273*: 76–86, 1942.
2. Hakomori, S. I. Aberrant glycosylation in cancer cell membranes as focused on glycolipids: overview and perspectives. Cancer Res., *45*: 2405–2414, 1985.
3. Carubia, J. M., Yu, R. K., Mascala, L. J., Kirkwood, J. M., and Varga, J. M. Gangliosides on normal and neoplastic melanocytes. Biochem. Biophys. Res. Commun., *120*: 500–504, 1984.
4. Hamilton, W. B., Helling, F., Lloyd, K. O., and Livingston, P. O. Ganglioside expression on human malignant melanoma assessed by quantitative immune thin layer chromatography. Int. J. Cancer, *53*: 1–8, 1993.

5. Tsuchida, T., Saxton, R. E., Morton, D. L., and Irie, R. F. Gangliosides of human melanoma. J. Natl. Cancer Inst., *78:* 45–54, 1987.
6. Houghton, A. N., Mintzer, D., Cordon-Cardo, C., Welt, S., Fliegel, B., Vadhan, S., Carswell, E., Melamed, M. R., Oettgen, H. F., and Old, L. J. Mouse monoclonal IgG3 antibody detecting G_{D3} ganglioside: a phase I trial in patients with malignant melanoma. Proc. Natl. Acad. Sci. USA, *82:* 1242–1246, 1985.
7. Cheung, N-K. V., Lazarus, H., Miraldi, F. D., Abramowsky, C. R., Kallic, S., Saarinen, U. M., Spitzer, T., Strandjord, S. E., Coccia, P. F., and Berger, N. A. Ganglioside G_{D2} specific monoclonal antibody 3F8: a phase I study in patients with neuroblastoma and malignant melanoma. J. Clin. Oncol., *5:* 1430–1440, 1987.
8. Irie, R. F., and Morton, D. L. Regression of cutaneous metastatic melanoma by intralesional injection with human monoclonal antibody to ganglioside G_{D2}. Proc. Natl. Acad. Sci. USA, *83:* 8694–8698, 1986.
9. Irie, R. F., Matsuki, T., and Morton, D. L. Human Monoclonal Antibody to Ganglioside G_{M2} for Melanoma Treatment. The Lancet, 786–787, 1989.
10. Livingston, P. O., Wong, G. Y., Adluri, S., Tao, Y., Padavan, M., Parente, R., Hanlon, C., Calves, M. J., Helling, F., Ritter, G., Oettgen, H. F., and Old, L. J. A randomized trial of adjuvant vaccination with BCG *versus* BCG plus the melanoma ganglioside G_{M3} in AJCC stage III melanoma patients. J. Clin. Oncol., in press, 1994.
11. Ritter, G., Boosfeld, E., Adluri, R., Calves, M. J., Oettgen, H. F., Old, L. J., and Livingston, P. O. Antibody response after immunization with ganglioside G_{D3} and G_{D3} congeners (lactones, amide and gangliosidol) in patients with malignant melanoma. Int. J. Cancer., *48:* 379–385, 1991.
12. Livingston, P. O., Natoli, E. J. Jr, Calves, M. J., Stockert, E., Oettgen, H. F., and Old, L. J. Vaccines containing purified G_{M2} ganglioside elicit G_{M2} antibodies in melanoma patients. Proc. Natl. Acad. Sci. USA, *84:* 2911–2915, 1987.
13. Livingston, P. O., Ritter, G., Srivastava, P., Padavan, M., Calves, M. J., Oettgen, H. F., and Old, L. J. Characterization of IgG and IgM antibodies induced in melanoma patients by immunization with purified G_{M2} ganglioside. Cancer Res., *49:* 7045–7050, 1989.
14. Eskola, J., Kayrty, H., Takala, A. K., Peltola, H., Ronneberg, P. R., Kha, E., Pekkanen, E., McVerry, P. H., and Makela, P. H. A randomized prospective field trial of a conjugate vaccine in the protection of infants and young children against invasive *Haemophilus influenzae* type b disease. N. Engl. J. Med., *323:* 1381–1387, 1990.
15. Anderson, P. Antibody response to *Haemophilus influenzae* type b and diphteria toxin induced by conjugates of oligosaccharides of the type b capsule with nontoxic protein CRM 197. Infect. Immun., *39:* 233–238, 1983.
16. Cahan, L. D., Irie, R. F., Singh, R., Cassidenti, A., and Paulson, J. C. Identification of a neuroectodermal tumor antigen (OFA-I-2) as ganglioside G_{D2}. Proc. Natl. Acad. Sci. USA, *79:* 7629–7633, 1982.
17. Ritter, G., Boosfeld, E., Calves, M. J., Oettgen, F. H., Old, L. J., and Livingston, P. O. Biochemical and serological characteristics of natural 9-*O*-acetyl G_{D3} from human melanoma and bovine buttermilk and chemically *O*-acetylated G_{D3}. Cancer Res., *50:* 1403–1410, 1990.
18. Ren, S., Scarsdale, J. N., Ariga, T., Zhang, Y., Klein, R. A., Hartmann, R., Kushi, Y., Egge, H., Yu, R. K. *O*-Acetylated gangliosides in bovine buttermilk. J. Biol. Chem., *267:* 12632–12638, 1992.
19. v. Nicolai, H., Müller, H. E., and Zilliken, F. Substrate specificity of neuraminidase from *Erysipelothrix rhusiopathiae*. Hoppe-Seyler's Z. Physiol. Chem., *359:* 393–398, 1978.
20. Kensil, C. R., Patel, U., Lennick, M., and Marciani, D. Separation and characterization of saponins with adjuvant activity from *Quillaja saponaria* molina cortex. J. Immunol., *146:* 431–437, 1991.
21. Dippold, W. G., Lloyd, K. O., Li, L. T., Ikeda, H., Oettgen, H. F., and Old, L. J. Cell surface antigens of human malignant melanoma: definition of six antigenic systems with monoclonal antibodies. Proc. Natl. Acad. Sci. USA, *77:* 6114–6118, 1980.
22. Criegee, R. The course of ozonization of unsaturated compounds. Rec. Chem. Prog., *18:* 111–120, 1957.
23. Wiegandt, H., and Baschang, G. Die Gewinnung des Zuckeranteils der Glykosphingolipide durch Ozonolyse und Fragmentierung. Z. Naturforsch., *20b:* 164–166, 1965.
24. Pappas, J. J., Keaveney, W. P., Gaucher, E., and Melvin, B. A new and convenient method for converting olefins to aldehydes. Tetrahedron Lett., *36:* 4273–4278, 1966.
25. Svennerholm, L. Quantitative estimation of sialic acids. II. Colorimetric resorcinol-hydrochloric acid method. Biochim. Biophys. Acta., *24:* 604–611, 1957.
26. Gray, G. R. The direct coupling of oligosaccharides to proteins and derivatised gels. Arch. Biochem. Biophys., *163:* 426–428, 1974.
27. Roy, R., and Lafferière, C. A. Michael addition as the key step in the synthesis of sialooligosaccharide protein conjugates from *N*-acroloylated glycopyranosyl-amines. J. Chem. Soc. Chem. Commun., 1709–1711, 1990.
28. Borch, R. F., Bernstein, M. D., and Durst, H. D. The cyanohydridoborate anion as a selective reducing agent. J. Am. Chem. Soc., *93:* 2897–2904, 1971.
29. Livingston, P. O., Koganty, R. R., Longenecker, B. M., Lloyd, K. O., and Calves, M. J. Studies on the immunogenicity of synthetic and natural Thomsen-Friedenreich (TF) antigens in mice: augmentation of the response by Quil A and SAF-m adjuvants and analysis of the specificity of the responses. Vaccine Res., *1:* 99–109, 1991.
30. Ritter, G., Boosfeld, E., Calves, M. J., Oettgen, H. F., Old, L. J., and Livingston, P. O. Antibody response after immunization with gangliosides G_{D3}, G_{D3} lactones, G_{D3} amide and G_{D3} gangliosidol in the mouse. G_{D3} lactone I indudes antibodies reactive with human melanoma. Immunobiology, *182:* 32–43, 1990.
31. Kanfer, J. N., and Hakomori, S. Sphingolipid biochemistry. *In:* D. J. Hanahan (ed.), Handbook of Lipid Research, Vol. 3, pp. 49–50. New York: Plenum Press, 1983.
32. Laine, R. A., Yogeeswaran, G., and Hakomori, S-I. Glycosphingolipids covalently linked to agarose gel or glass beads. J. Biol. Chem., *249:* 4460–4466, 1974.
33. Young, W. W., Jr., Laine, R. A., and Hakomori, S. An improved method for the covalent attachment of glycolipids to solid supports and macromolecules. J. Lipid. Res., *20:* 275–278, 1979.
34. Lowell, G. H., Ballou, W. R., Smith, L. F., Wirtz, R. A., Zollinger, W. D., and Hockmeyer, W. T. Proteosome-lipopeptide vaccines: enhancement of immunogenicity for malaria CS peptides. Science (Washington DC), *240:* 800–802, 1988.
35. Donnelly, J. J., Deck, R. R., and Liu, M. A. Adjuvant activity of the outer membrane protein complex of Neisseria meningitidis serogroup B for a polysaccharide-protein conjugate. Vaccines, *91:* 403–408, 1991.
36. Livingston, P. O., Calves, M. J., Helling, F., Zollinger, W. O., Blake, M. S., and Lowell, G. H. G_{D3}/proteosomes vaccine induce consistent IgM antibodies against the ganglioside G_{D3}. Vaccine, *11:* 1199–1204, 1993.
37. Apple, R. J., Domen, P. L., Muckerheide, A., and Michael, J. G. Cationization of protein antigens IV: increased antigen uptake by antigen presenting cells. J. Immunol., *40:* 3290–3295, 1988.
38. Tam, J. P. Synthetic peptide vaccine design: synthesis and properties of a high-density multiple antigenic peptide system. Proc. Natl. Acad. Sci. USA, *85:* 5409–5413, 1988.
39. Tam, J. P., and Lu, Y. Vaccine engineering: Enhancement of immunogenicity of synthetic peptide vaccines related to hepatitis in chemically defined models consisting of T- and B-cell epitopes. Proc. Natl. Acad. Sci. USA, *86:* 9084–9088, 1989.
40. Svennerholm, L. Chromatographic separation of human brain gangliosides. J. Neurochem., *10:* 613–623, 1963.